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Studies on fungi in a pinewood soil

V. — Root mycofloras of seedlings of *Pinus nigra* var. *laricio*

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INTRODUCTION

Living roots provide a discrete group of microhabitats for the study of fungal development and activity, and in the soil under study at Freshfield, Lancashire (BALASOORIYA & PARKINSON, 1967) pine roots make up an important component of the soil organic matter. The aim of the investigation reported here was to study the development of the root surface mycofloras of seedlings of *Pinus nigra* var. *laricio* in relation to age of the seedlings and to different parts of the root systems. During this investigation the ectotrophic mycorrhizas of the tree seedlings were not investigated.

MATERIALS AND METHODS

The pine seedlings studied were sampled from an area of natural tree regeneration in a plantation of *Pinus nigra* v. *laricio*. The soil and its mycoflora under this plantation have been described previously (BALASOORIYA & PARKINSON, 1967 ; PARKINSON & BALASOORIYA, 1967).

Initial studies were made to correlate seedling age (as determined by counting of annual rings in stem transverse sections) with morphological

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features easily recognizable in the field. Age determination by sectioning and microscopic observation was relatively simple, however, age determination in the field proved more difficult.

At the outset of this investigation it was decided to choose seedlings in their first, second and fifth year of growth. Seedlings in their first year of growth were easily recognized (bearing only one, terminal, whorl of needles); those in their second year of growth were also susceptible to macroscopic recognition; however, recognition of the age of older seedlings was more difficult — the classical whorl-counting technique being found to be unreliable. With practice a provisional selection of older seedlings was made on the height of the seedlings, after which whorls of needles and needle scars were counted and if it was then believed that the plant was of the desired age it was sampled, taken to the laboratory for precise ageing (by microscopic observation).

An attempt was made to determine seedling root development in the soil horizons (this being an A_1-C soil). To this end root systems were carefully uncovered and their main components mapped. As might be expected considerable variation in root development was observed, this variation was based on a tap-root structure (occasionally plants were found with a dichotomized tap root).

In the case of five year-old seedlings the root systems frequently extended to soil depths in excess of 30 cm (i.e. well into the C horizon) with horizontal and oblique lateral branches (up to 20 cm in length) passing through the F_2 and H layers of the A_0 and into the upper regions of the A_1 horizon.

In the study reported here the parts of the root system investigated were:

a. the upper part of the tap root (situated in the A_1 horizon);

b. the upper lateral branches (situated in the H layer);

c. the lower parts of the vertical root system.

ISOLATION OF FUNGI

The roots under study were transported to the laboratory where approximately 3 cm portions of the chosen parts of the root systems were subjected to the serial washing technique (HARLEY and WAID, 1954). Preliminary tests were made to determine the number of washings required to achieve optimum removal of detachable fungal propagules (data on which are provided in Figs. 1, 2 and 3), a brief analysis of this washing test data will be given later as it is believed to throw some light on fungi in the rhizosphere as well as providing the basis for the root surface studies. From these data 30 washings (each of 2 minutes duration) were chosen for dealing with roots of all ages.

After washing, excess moisture on the roots was removed using sterile filter paper, and the dried pieces of washed root were cut into 2 mm segments which were then plated onto acidified Czapek-Dox agar + yeast extract (4 root segments per plate).

Incubation of plates was at room temperature, colonies growing on the plates were sub-cultured and identification attempted.

In an attempt to ascertain the degree of colonizaion by fungi of the internal tissues of the roots, washed roots were dissected so that the cortex and stele were separated. Pieces of the cortex and stelar material were plated in the same way as were the whole root segments.

DIRECT OBSERVATION OF FUNGI

As well as the application of isolation techniques, direct observation of roots and the measurement of hyphal development on roots of different ages was attempted.

Washed roots were placed in lactophenol for between two and five days. They were then transferred to 0.5 % cotton blue in lactophenol and left overnight. Excess stain was removed by soaking the roots in lactophenol for two hours.

Pieces of epidermal tissue were dissected from five-year-old and two-year-old roots (pieces as large as 8 × 2 mm, free from adhering cortical material could be obtained). These pieces of epidermis were cut into pieces approximately 3 × 1.5 mm and were mounted on slides for microscopic observation.

In a preliminary study 30 pieces of epidermal tissue from 2 year old and 5 year old roots, and 20 pieces from 1 year old roots were stained and examined microscopically, the presence or absence of hyphae of various types (septate hyaline, septate dark, clump-bearing, very thin) on each piece was noted.

To attempt standardization of the technique, a 2.5 cm length of upper taproot was dissected to yield 5 pieces of epidermal tissue (each piece being 3 × 1.5 mm). These 5 pieces were mounted in series in lactophenol and each was observed under the microscope. Ten random fields of view (high power, × 400) were examined on each of the 5 epidermal pieces. The total mycelial length in each of these fields of view was drawn with the aid of a camera lucida apparatus. Measurements of these drawings using a map-measuring device, plus knowledge of the degree of magnification and area of each field of view, allowed calculation of hyphal lengths on known areas of root surface.

RESULTS AND DISCUSSION

1. Analysis of washing data.

The washing data (Fig. 1) for roots from the A_1 horizon show an increase in the number of colonies on dilution plates prepared from washing waters after the 20th washings. This effect was noted in several tests and therefore it was decided to record the fungal species removed from the roots at different stages in the washing procedure. These data are given in Figures 2 and 3, and demonstrate the increase in the number of fungal propagules removed from the roots after about 20 washings.

From the data it can be seen that the increase in removed propagules is an effect of the removal of sterile dark fungi and *Penicillium* spp.

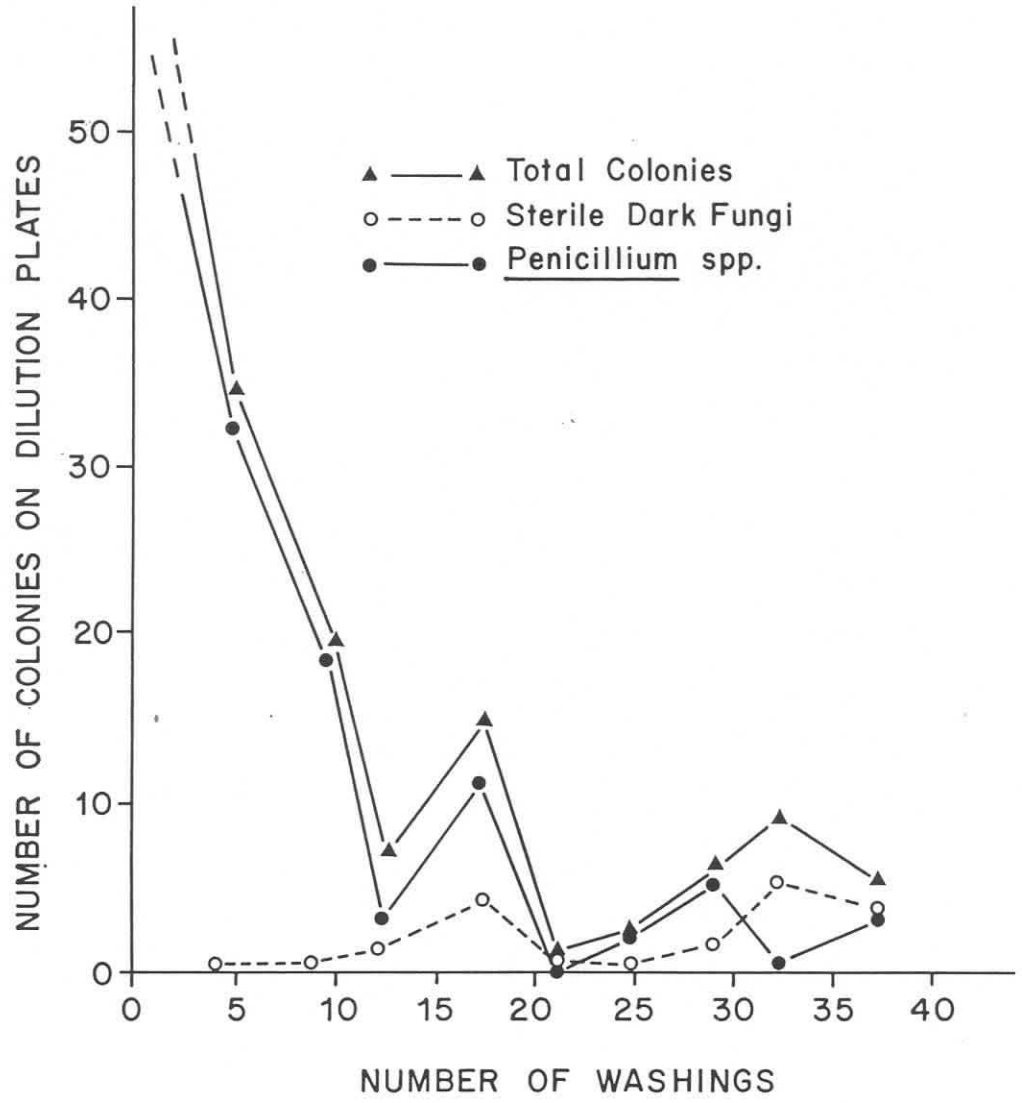

FIG. 1. — Numbers of fungal propagules removed from roots of *P. nigra* growing in the A_1 horizon by the Harley and Waid (1955) technique. (Expt. 1).

In the case of 5-year-old plants (and, to a lesser extent, 2-year-old plants), visible pieces of epidermal tissue were detached from the roots during the washing process. This effect is reflected in the large numbers of colonies of sterile dark fungi appearing on dilution plates prepared from the 5-year-old plant root washing water. In the case of 1-year-old roots no detaching of epidermal tissue was observed and no sterile dark fungi appear on the dilution plates prepared from the washing waters.

2. **Comparison of root surface mycofloras of Pine seedlings in their first, second, and fifth years of growth.**

For this comparison, portions of the upper tap root of seedlings of all ages were sampled, i.e., the oldest portions of each root was sampled, these portions all being from the same soil horizon.

TABLE I

Percentage frequency of occurences of fungi on the upper tap root seedlings of *P. nigra* of different ages (figures indicates % segments colonized).

FUNGI ISOLATED	AGE OF SEEDLINGS		
	1 YEAR	2 YEARS	5 YEARS
Sterile dark fungi SDF	11.0	23.0	60.5
SDG	—	14.0	4.0
X3	—	—	3.5
Z11	2.5	—	2.5
Mortierella isabellina	1.5	—	2.5
marburgensis	1.0	3.5	4.0
parvispora	0.5	3.0	—
Other Mortierella species	2.0	—	—
Absidia coerulea	—	—	0.5
Cylindrocarpon cylindroides	4.5	—	—
radicicola	4.0	—	1.0
Gliomastix murorum	1.0	2.0	—
Oidiodendron fuscum	5.5	—	—
Penicillium decumbens	27.5	7.5	9.0
lividum	0.5	6.0	2.0
Other Penicillium species	16.0	24.5	19.5
Phialophora fastigiata	—	3.0	—
Phoma sp.	0.5	0.5	—
Aureobasidium pullulans	—	0.5	—
Rhizoctonia sp.	—	—	0.5
Trichoderma viride	6.5	6.0	6.0
Verticillium sp.	—	—	1.0
Z8	14.5	10.0	—
Clamp-bearing hyphae	—	—	0.5
Sterile root segments	0.5	2.5	1.0
Total root segments plated	200	200	200

The results of this comparison are given in Table 1. From these data several facts emerge:

a. The sterile dark form (SDF), which bore a marked resemblance to *Mycelium radicis atrovirens,* increased in frequency of occurrence on the roots with increasing age of the roots. The regularity of isolation of this fungus from upper tap roots of 5-year-old plants is shown in Table I.

b. From the youngest roots (1-year-old) *Penicillium decumbens* was the most frequently isolated species. This fungus was isolated with considerably reduced frequency from older roots (with increasing root age all Penicillium species showed decreased frequency of occurrence).

c. Members of the Mucorales did not appear to vary significantly in frequency of occurrence with increasing root age.

d. Between the age groups there was a considerable difference in the number of species of colonizing fungi which achieved the status of « most frequently isolated species », with increasing age this number declined (Table II indicates that in 5-year-old roots the sterile dark form, SDF, was the most frequently isolated species on 8 out of 10 plates).

TABLE II

Fungi isolated from the upper tap roots of ten 5-year-old plants (figure represent % frequency of occurrence; numerous plated root segments yielded more than one fungus).

FUNGI ISOLATED	PLANT NUMBER									
	1	2	3	4	5	6	7	8*	9	10
Sterile dark fungi SDF	45	100	35	55	90	30	90	30	40	90
SDG	20					20				
X3				35						
Absidia coerulea			5							
Mortierella marburgensis ..	30					10				
isabellina	5		5			15				
Cylindorcarpon radicicola ..				5	5					
Penicillium decumbens			35	10	10	20	5			10
lividum	15					5				
spp.	30		60			45		10	35	5
Rhizoctonia sp.										5
Trichoderma viride			5			5		30	20	
Verticillium sp.							5			
Clamp-bearing hyphae				5	5					
Segment yielding no fungi..	10								5	

* The low number of colonies arising from plated root segments of plant 8 was due to rapid overgrowth by *Trichoderma viride*.

Thus there was a decline in variation in the root surface mycofloras with increasing age of the plants. Data given in Table III indicates this decline in variation.

TABLE III

Number of plants in each group on whose roots each fungus was « most frequently isolated ».

MOST FREQUENTLY ISOLATED SPECIES	AGE GROUP		
	1 year plants	2 year plants	5 year plants
Sterile dark fungi SDF	1	3	7 + 1 =
SDG		2	
Z8	2 + 1 =	1	
Z11		1	
Penicillium decumbens	2 + 2 =		1 =
Penicillium sp.		1	
Trichoderma viride	1	1	
Phialophora fastigiata		1	
Cylindrocarpon radicicola	1 =		
cylindroides	1		
Oidiodendron fuscum	1		

From these data some indications is given of fungal succession on the roots of *Pinus nigra* :

Roots of 1-year-old plants — mycoflora closely resembles that of the soil, with *Penicillium decumbens* as the most frequently isolated species.

Roots of 5-year-old plants — mycoflora more stable and characterized by an apparent dominance of the sterile dark form (SDF).

Several species (*Phoma* spp. ; *Gliomastix murorum* and *Phialophora fastigiata*) isolated from portions of root systems situated in the A_1 horizon had previously only been isolated from C horizon soil (PARKINSON and BALLASOORIYA, 1967).

3. Comparison of root surface mycofloras of upper tap, lower vertical, and upper lateral roots of 5-year-old pine seedlings.

In a study of this type several variables must be considered: the difference in fungi available for root colonization (a result of the growth of the root system through the A_1 horizon into the C horizon, the mycofloras of these horizons have been described by PARKINSON and BALASOORIYA, 1967); the

possibility of marked local variations in available fungi within each horizon (PARKINSON and BALASOORIYA, 1969); the difference in age of the different parts of the root system (with consequent differences in stages of microbial succession).

Table IV gives data on the fungi isolated from the three zones. From these data it would seem (at least in terms of frequency of occurrence of sterile dark fungi) that the lower vertical roots (i.e. those approximately one-year-old) resemble older upper tap roots (5-year-old) more than the roots of seedlings in their first year of growth (Table I). This would appear to indicate the possibility of hyphal colonization of young developing roots from older parts

TABLE IV

Percentage frequency of occurrence of fungi on upper tap roots, lower vertical roots and upper lateral roots of five year old seedlings of *P. nigra* (figures indicate % segments colonized).

FUNGI ISOLATED	UPPER TAP ROOTS	LOWER VERTICAL ROOTS	UPPER LATERAL ROOTS
Sterile dark fungi SDF	40.0	20.0	50.0
SDG	6.0	—	—
SDA	—	20.0	—
Z11	2.0	15.0	3.0
HS	—	—	14.0
Mortierella isabellina	4.0	3.0	—
marburgensis	6.0	—	—
vinacea	—	1.0	—
Absidia coerulea	—	1.0	—
Asperigillus sp.	1.0	1.0	—
Cylindrocarpon cylindroides	—	7.0	—
radicicola	—	10.0	—
Penicillium decumbens	1.0	2.0	20.0
lividum	1.0	1.0	2.0
Other Penicillium species	20.0	5.0	7.0
Phialophora fastigiata	—	7.0	—
Phoma sp.	1.0	—	—
Rhizoctonia sp.	1.0	—	—
Sporormia sp.	1.0	—	—
Trichoderma viride	1.0	—	5.0
Verticillium sp.	—	1.0	—
Clamp-bearing forms	15.0	—	—
Number of segments plated	200	200	200

of the root system as well as from the surrounding soil. That root colonization from the surrounding soil still occurs is indicated by the presence of species (i.e., *Cylindrocarpon* spp. and *Phialophora fastigiata*) which are restricted to the C horizon.

In the case of the upper lateral roots comparable effects are seen (Table IV), indeed the sterile form SDF is here a more prominent root colonizer.

4. **Penetration of roots by fungi — results of root dissection.**

Data on fungi isolated from plating the products of root dissection of 1, 2 and 5-year-old roots of *P. nigra* are given in Table V. These data indicate a low degree of penetration of the stele, with a lower degree of penetration of steles of the older roots. This latter phenomenon might be the result of the greater ease with which cortical tissue could be removed from the stelar material (i.e. it is likely that the data for 1 and 2 year roots may result from the contamination of pieces of stele with small amounts of adhering cortex).

TABLE V

Fungi isolated from dissected upper tap roots of 1, 2 and 5 year seedlings of *P. nigra* (figures represent % frequency of occurrence).

	1 Year Plants		2 Year Plants		5 Year Plants	
	Cortex	Stele	Cortex	Stele	Cortex	Stele
Sterile dark fungi SDF	17	17	36	31	60	—
SDG	—	—	11	—	5	—
Z11	—	6	11	—	5	7
SDA	29	—	—	—	—	—
Mortierella isabellina	—	—	—	—	—	—
marburgensis	3	—	—	—	2	—
Aspergillus sp. 6	6	—	—	—	—	—
Cylindrocarpon cylindroides	6	—	3	—	—	—
Radicicola	3	—	—	—	—	—
Penicillium decumbens	28	—	6	—	8	—
Penicillium spp.	14	—	40	—	20	—
Phoma sp.	—	—	3	—	—	—
Trichoderma viride	—	—	—	—	2	—
Basidiomycete spp.	—	3	—	11	2	3
Segments overgrown by bacteria	—	10	3	3	—	—
Uncolonized segments	7	64	—	55	—	90

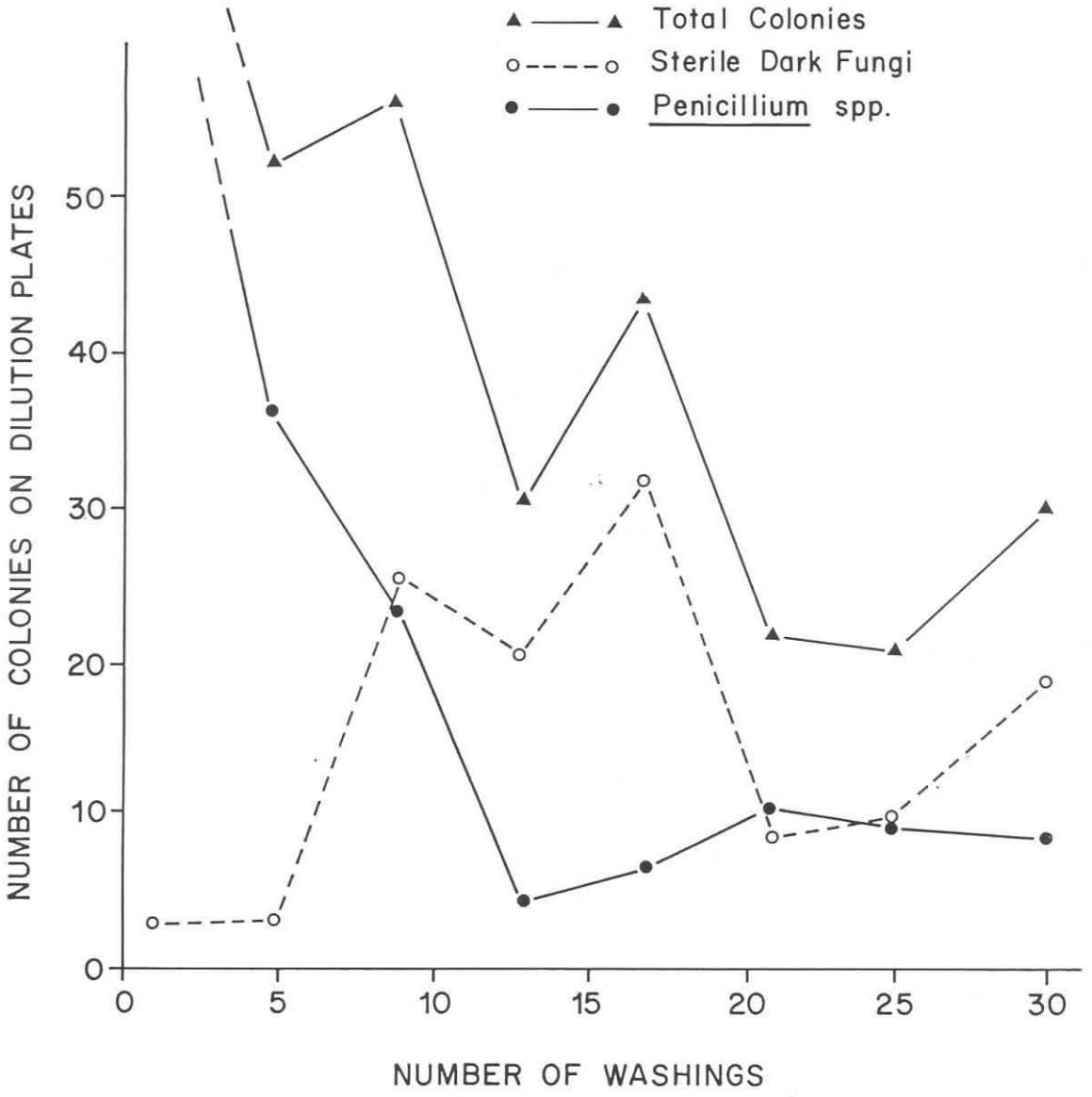

FIG. 2. — Numbers of fungal propagules removed from roots of *P. nigra* growing in the A_1 horizon by the Harley and Waid (1955) technique. (Expt. 2).

5. **Direct observation of root surfaces.**

a. *Preliminary observations.*

The observation of 30 epidermal segments from each root age group allowed distinction of several types of fungal hyphae. These preliminary data are given in Table VI and indicate the feasibility of this type of observation (although the sample size in this investigation was too small to allow proper comparisons between colonization of roots of different ages).

b. *Hyphal measurements.*

This technique was applied to 3 plants in their second year of growth and to 3 five-year-old plants. Fifty areas of known size were observed (these areas were microscope fields) and hyphal lengths were measured. From these data the amount of hyphal development per square millimeter of root surface was calculated and these data are given in Table VII.

TABLE VI

Types of hyphae observed on epidermal segments of washed upper tap root from seedlings of *P. nigra* in their first, second et fifth year of growth.

(Number indicate the number of segments on which each hyphal class was observed. Total number of segments observed was 30 in the case of 1 and 2 year seedlings and 20 in the case of 5 year seedlings.)

	Thin Hyphae	Dark Septate Hyphae	Hyaline Septate Hyphae	Asep-tate Hyphae	Clamp-bearing Hyphae	No Hyphae Visible
1 year seedlings (30) ..	6	16	10	2	0	3
2 year seedlings (30) ..	11	23	10	5	5	3
5 year seedlings (30) ..	13	28	8	3	6	1

TABLE VII

Hyphal development on surface of upper tap roots of 3 and 5 year seedlings of *P. nigra*.

	Length of hyphae (mm) per mm² of root surface		
	Plant 1	Plant 2	Plant 3
2 year plants	9.23	12.74	11.07
5 year plants	10.06	14.84	12.26

A combination of isolation and direct observation techniques applied to root mycofloras of *Pinus nigra* seedlings have provided data indicating the predominance of the sterile dark form (SDF) on the roots. The fungus (SDF) is believed to be *Mycelium radicis atrovirens* (Melin). SDF was replaced as a dominant form on certain 5 year roots by a Basidiomycete, and clamp-bearing hyphae were observed regularly during direct observations of segments of root epidermis.

The importance of *Mycelium radicis atrovirens* on tree roots has been discussed by Robertson (1954) and Harley and Waid (1955). The relationship of this fungus to *Pinus* spp. has been discussed by Rayner and Neilson-Jones (1944), Robertson (1954), Levisohn (1954), and Harley (1959), from these discussions a complex picture emerges. Under pure culture conditions *M. radicis atrovirens* has very marked pathogenic properties towards *Pinus sylvestris*; however, it can achieve dominance on root surfaces of healthy elongating roots of pines growing under natural conditions (Robertson, 1954), when its pathogenic effects appear limited to a small degree of pseudomycorrhizal association (Robertson, 1954; Harley, 1959). Robertson (1954) consi-

dered that, in natural conditions, *M. radicis atrovirens* was capable of attacking pine roots under special physiological conditions (i.e. root senescence), and that it is not pathogenic to healthy roots in natural soils.

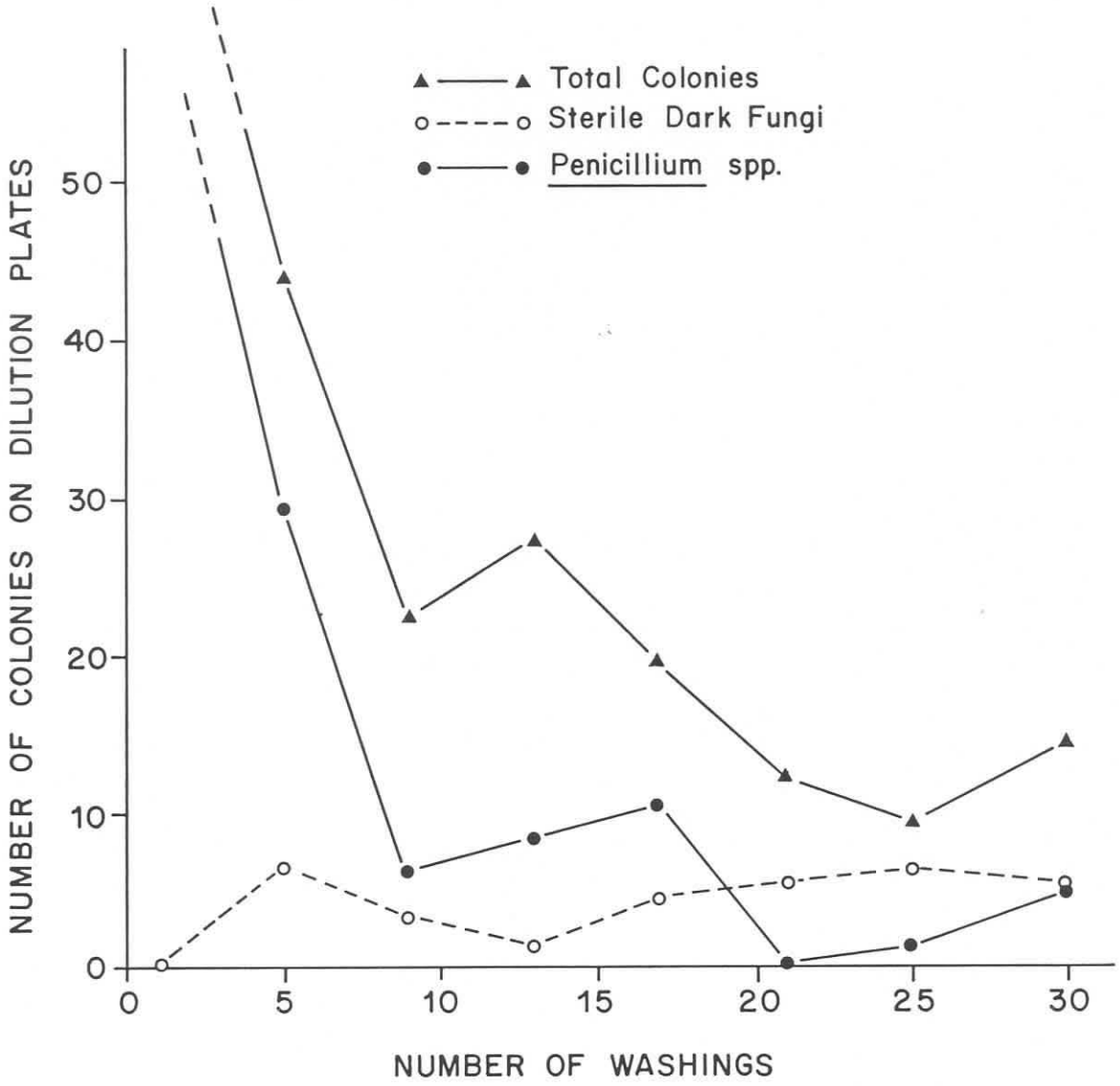

FIG. 3. — Numbers of fungal propagules removed from roots of *P. nigra* growing in the C horizon by the Harley and Waid (1955) technique.

The results obtained in this study correlate with those provided by ROBERTSON (1954), given that SDF is equitable with *M. radicis atrovirens*. The sterile dark form (SDF) is a dominant form on root surfaces of *Pinus nigra laricio* seedlings and also in the root cortex, but shows a low degree of penetration into the stelar tissues.

Evidence for annual agricultural crops has not indicated colonization of young roots by hyphal growth from older parts of the root (TAYLOR & PARKINSON, 1961). SEWELL (1959), studying *Calluna*, considered that the vertical distribution of fungi on root surfaces reflected that in the surrounding soil and found no evidence of hyphal growth down roots. However, evidence of

limited root colonization by hyphal growth on beech roots was found by HARLEY and WAID (1955). In this study, the possibility of colonization of young roots partly by hyphal growth from older parts of the root system has been suggested. Vertical distribution of fungi on the roots, mirroring that in the soil profile, was seen, but upon this was superimposed the effect of hyphal down-growth on the root surfaces.

SUMMARY

Application of isolation and direct observation techniques have been given data on funga successions on the root surface of *Pinus nigra laricio* seedlings with increasing root age. The role of sterile dark fungi as root surface colonizers has been emphasized.

Young roots of older seedlings appeared to be colonized partly from the surrounding soil and partly by hyphal downgrowth from older parts of the root system.

Results from root dissection studies indicated a low degree of stelar penetration by sterile dark forms in the young roots sampled.

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